

Effects of Temperature and Salinity on the Survival of *Vibrio vulnificus* in Seawater and Shellfish

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Sterilized seawater was used to assess the effects of temperature and salinity on the survival of *Vibrio vulnificus*. In the temperature range of 13 to 22°C, numbers of *V. vulnificus* increased during the 6-day incubation. Temperatures outside this range reduced the time of *V. vulnificus* survival in sterile 10-ppt seawater. At these restrictive temperatures, *V. vulnificus* numbers were reduced by 90% after 6 days of incubation. Incubation between 0.5 and 10.5°C demonstrated that *V. vulnificus* survives poorly below 8.5°C. At salinities between 5 and 25 ppt and at 14°C, *V. vulnificus* numbers actually increased or remained unchanged after 6 days of incubation. At salinities of 30, 35, and 38 ppt, numbers of *V. vulnificus* decreased 58, 88, and 83%, respectively. *V. vulnificus* could not be recovered from deionized water, indicating lysis. When a rifampin-resistant strain of *V. vulnificus* was used to inoculate sterilized and unsterilized seawater (20 ppt, 20°C), numbers increased in sterile seawater but decreased to undetectable levels in 14 days in the unsterilized seawater, indicating that biological factors may play a role in the survival of *V. vulnificus* in the environment. Since our studies demonstrated sensitivity to low temperatures, the survival of *V. vulnificus* in naturally contaminated oysters at temperatures of 0, 2, and 4°C was also determined. Numbers of endogenous *V. vulnificus* in oyster shellstock increased by more than 100-fold in shellstock stored at 30°C but were reduced approximately 10- and 100-fold after 14 days at 2 to 4°C and 0°C, respectively. We conclude that both biological and physicochemical factors are important to the survival of *V. vulnificus* in the environment and that temperature is critical to controlling its growth in oyster shellstock.

Vibrio vulnificus, an autochthonous member of the estuarine flora, is remarkably virulent for individuals who are immunocompromised or have liver dysfunctions which result in increased levels of iron in serum (2, 25). Although the consumption of raw oysters has been the primary vehicle for food-borne transmission of *V. vulnificus* (2, 9, 25), individuals have become infected through wounds which come in contact with marine waters (2, 24). *V. vulnificus* is most commonly found in estuarine and marine waters, particularly of the Gulf Coast and other temperate regions (10, 19, 20, 28, 29), and can be found in high numbers in oysters from these waters (3, 23, 26, 27).

Surveys of U.S. coastal waters and results from epidemiological studies indicate that warm water temperatures (>20°C) and low-to-moderate salinities are associated with the presence of *V. vulnificus* (10, 19, 28, 29). The optimal temperature and salinity for growth of *V. vulnificus* have been determined (12), and other studies suggest that temperature and salinity are controlling factors in its distribution (18, 29). Low temperatures have been shown to be detrimental to survival and induce formation of viable but nonculturable cells (17, 31). Although Nilsson et al. (16) reported that nonculturable *V. vulnificus* resuscitated to a culturable form when cells were shifted to a favorable temperature, subsequent studies have found that this reversion was not resuscitation but the replication of a few culturable cells (21, 30).

Therefore, the significance of this survival form in the environment remains debatable.

Likewise, there are conflicting reports on the response of *V. vulnificus* in oyster shellstock to abuse temperatures. Murphy and Oliver (15) reported that the numbers of *V. vulnificus* in artificially inoculated oyster shellstock stored at 22°C decreased 1,000-fold, whereas others have reported increases in numbers at similar temperatures when examining natural *V. vulnificus* populations in oyster shellstock (5, 7, 11, 26).

These studies were conducted to further elucidate and define the survival properties of *V. vulnificus* in the environment and oysters. Natural seawater samples were used to determine the effects of temperature, salinity, and the estuarine microbiota on survival. Endogenous *V. vulnificus* present in oysters was monitored to ascertain how low and high storage temperatures impact their survival and numbers in oyster shellstock.

MATERIALS AND METHODS

Strains and inoculum preparation. *V. vulnificus* 4793, 4832, and 4965 are from oysters harvested from estuaries of the Gulf of Mexico, and ATCC 27562 is a human isolate. Long-term storage of cultures was in liquid nitrogen. Cultures were grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing an additional 1% NaCl (TSA-NaCl) (10 g of NaCl per liter of TSA) and incubated overnight at room temperature (20 to 25°C). Following incubation, the *V. vulnificus* growth was harvested, suspended, and washed three times (8,000 × g, 20 min, 25°C) with 10-ppt sterile seawater or phosphate-buffered saline (PBS) (6). Suspensions were then adjusted to an A_{420} of 0.64 (2.0×10^8 CFU/ml) with 10-ppt sterile seawater or PBS.

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When a *V. vulnificus* pool consisting of strains 4793, 4832, and ATCC 27562 was used as the inoculum, suspensions of each strain were adjusted to an A_{420} of 0.64 and mixed in equal parts.

A rifampin (RIF) (Sigma Chemical Co.)-resistant strain of *V. vulnificus* ATCC 27562 was isolated by spread plating 1.0 ml of a suspension containing approximately 10^{10} CFU/ml on TSA-NaCl containing 100 μ g of RIF per ml. TSA-NaCl-RIF was prepared by adding an appropriate quantity of a 100 \times RIF stock solution in methanol to sterile TSA-NaCl at 47°C. Inoculated plates were incubated at room temperature for 48 h, and several of the resulting colonies were streaked on TSA-NaCl-RIF plates. Prior to inoculation, strain VV^{RIF} was streaked on consecutive days on TSA-NaCl-RIF to ensure RIF resistance. Separate experiments showed that the growth rate of VV^{RIF} was identical to that of the parent strain ATCC 27562.

Seawater preparation and inoculation. Samples were prepared by using subsurface seawater (38 ppt), aseptically collected from the Gulf of Mexico, that was diluted with deionized water to the appropriate salinity and sterilized at 121°C for 15 min. Salinity was determined by measuring the refractive index. A standardized bacterial suspension was added to sterilized seawater to reach a final concentration of around 2.0×10^4 CFU/ml. To determine the optimal temperature range for survival, triplicate tubes containing 25 ml of the *V. vulnificus* suspension in 10-ppt (a noninhibitory salinity) sterile seawater were incubated with agitation in a temperature gradient incubator. The temperature gradient was monitored daily in blank tubes at each end of the incubator block by using a thermometer to measure seawater temperature. Temperatures did not vary by more than 1°C. Likewise, the optimal range of salinity was determined in triplicate tubes incubated with agitation at 14 and $21 \pm 1^\circ\text{C}$, noninhibitory temperatures. The remainder of the samples were set up in duplicate by using 500-ml screw-cap Erlenmeyer flasks containing 300 ml of sterile seawater at the appropriate salinity.

Bacterial enumerations. At each time point, a sample was removed from each seawater sample, and a portion was plated on TSA-NaCl directly or following dilution in 0.1% peptone. The numbers of CFU/milliliter were determined after incubation at room temperature (20 to 25°C) for 20 to 24 h. Mean values for replicate samples were determined. Survival (percent CFU/milliliter) was calculated by using the numbers of CFU/milliliter immediately following inoculation as 100%.

Natural flora microcosms. Samples were prepared in duplicate and contained 250 ml of unsterilized seawater (20 ppt) in screw-cap Erlenmeyer flasks (capacity, 500 ml). Subsurface seawater was collected aseptically at the mouth of Mobile Bay and used within 24 h of collection. One set of samples contained unsterilized seawater only, a second set contained unsterilized seawater plus VV^{RIF}, and the third set contained sterilized seawater (121°C and 15 lb/in² for 15 min) plus VV^{RIF}. The samples were incubated at 20°C with gentle shaking. The numbers of CFU/milliliter were determined with TSA-NaCl-RIF following incubation at room temperature for 24 h.

Oyster studies. Oysters (*Crassostrea virginica*) were collected from coastal waters of Louisiana and transported on ice to the laboratory for storage studies. Shellstock was then transferred to open plastic bags and placed in water maintained at 0, 2, 4, and 30°C. The tops of the bags remained open during storage. Lower incubation temperatures (0 to 4°C) were obtained by using salt water and a cooling core

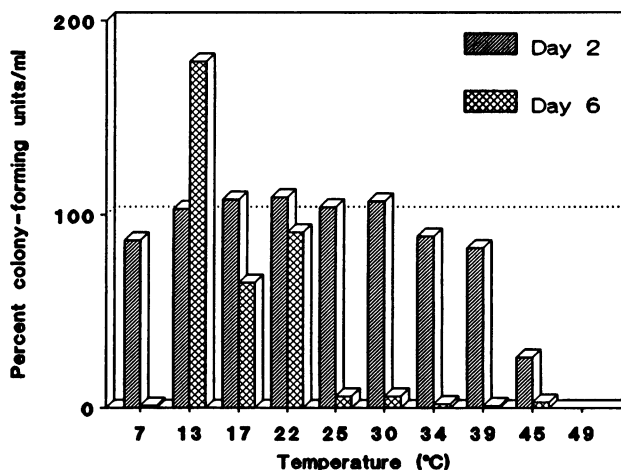


FIG. 1. Effects of temperature on the survival of *V. vulnificus* 4965 in 10-ppt sterile seawater. Samples were incubated in a temperature gradient water bath. Mean percentages and the mode temperatures for three tubes are shown. Percentages were calculated by using the number of CFU/milliliter following inoculation as 100%.

(Bio-Rad, Richmond, Calif.) connected to a recirculating refrigerated water bath (Pharmacia LKB, Piscataway, N.J.) filled with polyethylene glycol. The water temperatures were monitored daily and were within 0.5°C of the value reported. After various periods of storage over 14 days, with initial sampling 9 h after harvest, oysters were removed from the bag and the shells were scrubbed with a brush under running water and opened with a shucking knife. Separate oysters were used to measure the internal temperature, which was typically 2 to 3°C greater than the storage water temperature. The meats and liquor were then transferred to sterile blender jars. Approximately 150 g of oyster meats (10 oysters) was homogenized with an equal weight of sterile PBS (6), and dilutions of 10^{-1} to 10^{-6} were prepared. To enumerate *V. vulnificus*, a three-tube most-probable-number test using alkaline peptone water (6) enrichment was used. Alkaline peptone water was inoculated with 1.0-ml samples of the appropriate dilutions and incubated for 12 h at 35°C. Tubes of alkaline peptone water exhibiting turbidity were streaked on modified colistin-polymyxin B-cellobiose agar (27) and incubated for 18 to 20 h at 39°C. *V. vulnificus*-like colonies were confirmed by using a specific monoclonal antibody (FRBT37), as previously described (27).

RESULTS AND DISCUSSION

Effect of temperature on survival. Survival of *V. vulnificus* 4965 was optimal at temperatures between 13 and 22°C in 10-ppt (noninhibitory salinity) sterile seawater (Fig. 1). On day 2 numbers of *V. vulnificus* had increased in the temperature range of 13 to 30°C, but on day 6 the greatest numbers were found in the temperature range of 13 to 22°C. Temperatures outside this range (i.e., 25 to 45°C) reduced the time of *V. vulnificus* survival. At these restrictive temperatures, *V. vulnificus* numbers were reduced by 90% after 6 days of incubation.

To corroborate the findings with strain 4965, a pool of *V. vulnificus* strains (4793, 4832, and ATCC 27562) was used to inoculate samples of sterile seawater (10 ppt) that were incubated at 5, 12, 20, and 35°C. As was observed with *V.*

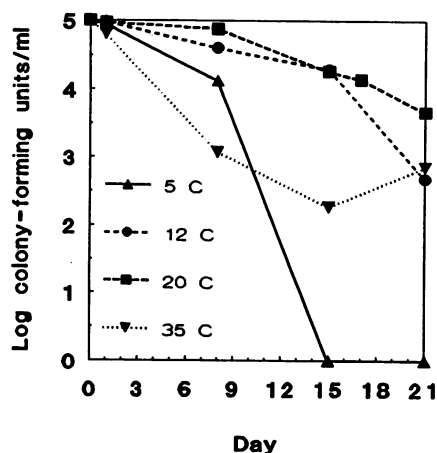


FIG. 2. Survival of a pool of three *V. vulnificus* strains (4793, 4832, and ATCC 27562) in 10-ppt sterile seawater. The data are mean values from duplicate flasks.

vulnificus 4965, the pool of *V. vulnificus* strains was sensitive to low and high temperature extremes (Fig. 2). The most rapid decrease in numbers of *V. vulnificus* occurred in samples incubated at 5 and 35°C. No organisms were recovered from samples incubated at 5°C after 15 days of incubation. For samples held at 35°C, the CFU of *V. vulnificus* (7.2×10^2 /ml, log = 2.86) were reduced by more than 2.5 logs. Survival was best at 20°C; at this temperature, the CFU (4.7×10^3 /ml, log = 3.67) declined at the slowest rate and had a reduction of 1.3 logs after 21 days. The final number of CFU (4.8×10^2 /ml, log = 2.68) in samples incubated for 21 days at 12°C was slightly less than the number in samples incubated at 35°C (7.2×10^2 /ml, log = 2.86); however, the decline in *V. vulnificus* counts in the samples at 12°C was much slower than the decline in those incubated at 35°C (Fig. 2).

To further define the sensitivity of *V. vulnificus* to low temperatures, a more detailed study was conducted with samples held at temperatures from 0.5 to 10.5°C and at a salinity of 10 ppt. A decrease in temperature correlated with a decrease in the time of survival (Fig. 3). In samples incubated at temperatures between 0.5 and 6.0°C, the numbers of *V. vulnificus* were 31 to 66% of the initial concentration after 3 days of incubation, 7 to 29% after 7 days, and 0 to 8% after 13 days. At temperatures of 8.5°C and higher, the numbers of *V. vulnificus* increased and remained higher for the duration of the experiment compared with the initial concentration of cells (Fig. 3). The sensitivity of *V. vulnificus* to low temperature is consistent with the results of environmental surveys (10, 19, 28, 29) and studies of other vibrios (4). The increase in CFU/milliliter in the absence of a nutrient, although not reported for *V. vulnificus*, has been observed with other *Vibrio* spp. in response to starvation (14).

Effects of salinity on survival. The survival of *V. vulnificus* 4965 was determined in seawater samples with salinities between 0 and 38 ppt that were incubated at 14 and 21°C, noninhibitory temperatures. In samples with salinities between 5 and 25 ppt (14°C), *V. vulnificus* numbers increased during the 6 days of incubation with the exception of samples containing 10-ppt seawater, in which numbers decreased by 13% (Fig. 4). In comparison, at salinities of 30, 35, and 38 ppt, numbers of *V. vulnificus* decreased by 58, 88, and 83%, respectively. Although *V. vulnificus* numbers in all samples

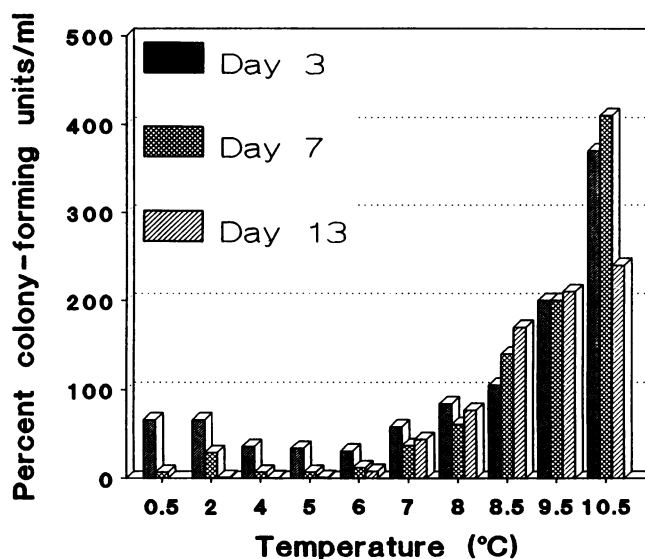


FIG. 3. Survival of a pool of three *V. vulnificus* strains (4793, 4832, and ATCC 27562) in 10-ppt sterile seawater at temperatures from 0.5 to 10.5°C. The data are mean values from duplicate tubes.

were reduced by >87% after 16 days of incubation, samples with salinities between 5 and 25 ppt contained the highest numbers of CFU. *V. vulnificus* could not be recovered from deionized water (0 ppt), probably because of lysis. Over the same range of salinities but at an incubation temperature of 21°C, numbers of *V. vulnificus* remained unchanged or dropped slightly (<0.5 log) in all samples after 2 days of incubation (data not shown). Samples with salinities between 5 and 15 ppt contained the greatest number of *V. vulnificus* after 6 days of incubation, but numbers in all samples, regardless of the salinity, dropped 1 to 2 logs. Both temperatures used in the salinity studies, 14 and 21°C, were in the optimal temperature range for survival (Fig. 1); however, the numbers of *V. vulnificus* remained higher at 14°C over a broader range of salinities (5 to 25 ppt; Fig. 4)

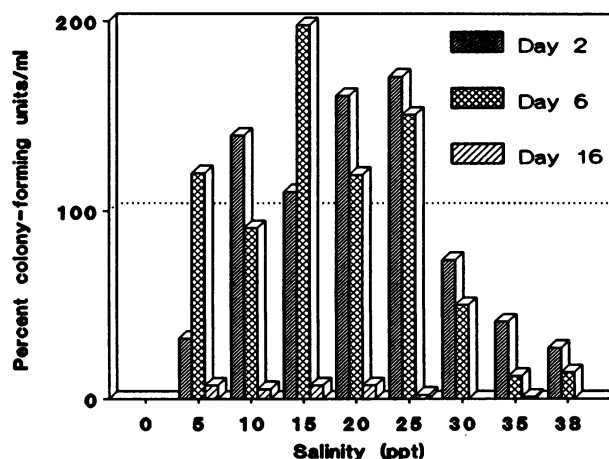


FIG. 4. Effects of salinity on the survival of *V. vulnificus* 4965 in sterile seawater incubated at 14°C. Mean percentages for triplicate tubes are shown. Percentages were calculated by using the number of CFU/milliliter following inoculation as 100%.

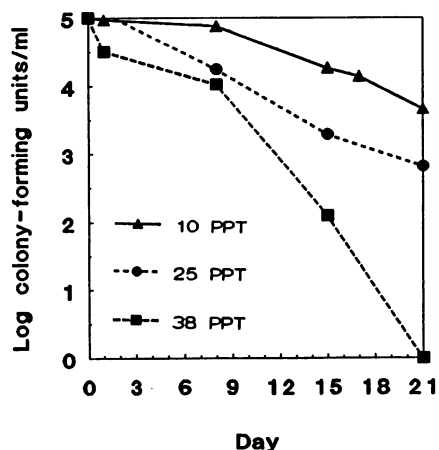


FIG. 5. Effects of salinity on the survival of a pool of three *V. vulnificus* strains (4793, 4832, and ATCC 27562) in sterile seawater incubated at 20°C. Mean values from duplicate flasks are shown.

than at 21°C (data not shown). These results indicate that temperature may influence the tolerance of *V. vulnificus* to unfavorable salinities.

Salinity studies were repeated by using a pool of three *V. vulnificus* strains (4793, 4832, and ATCC 27562). Studies were conducted at 20°C, because this temperature is easily exceeded in Gulf Coast estuaries during the summer months but is still within the optimal range for survival (13 to 22°C; Fig. 1). Among samples containing either 10-, 25-, or 38-ppt sterile seawater, survival of *V. vulnificus* strains was greatest in samples containing 10-ppt seawater, for which the rate of decline was the slowest and the final CFU/milliliter after 21 days of incubation was the highest (4.7×10^3 /ml, log = 3.67; Fig. 5). Samples with salinities of 25 and 38 ppt declined faster and had lower final numbers of *V. vulnificus* after 21 days of incubation, 6.7×10^2 (log = 2.82) and 0 CFU/ml, respectively. Therefore, salinities of 25 ppt and higher can have an impact on survival, although the effects were not as dramatic as those exhibited by temperature.

Survival of RIF-resistant *V. vulnificus*. A RIF-resistant isolate of parent strain ATCC 27562 was used to assess the impact of biological factors on the survival of *V. vulnificus* in natural seawater. Survival of VV^{RIF} was affected by factors other than temperature and salinity (20°C, 20 ppt) in non-sterilized seawater (Fig. 6). Numbers of VV^{RIF} declined until no CFU were detected after 14 days. In contrast, numbers of VV^{RIF} increased by 2 logs in sterile seawater (Fig. 6). Control samples containing only untreated seawater did not yield any CFU on TSA-NaCl-RIF. Bacteriophage and protozoa have been reported to play a role in the survival of enteric bacteria in marine waters (13, 22) and may be involved in the decline in VV^{RIF} numbers in the untreated seawater. In estuarine environments, *V. vulnificus* may be protected from predation by its association with oysters (26).

Effects of temperature on *V. vulnificus* numbers in oyster shellstock. Storage of oyster shellstock at 0, 2, and 4°C caused 1-log reductions in the numbers of natural *V. vulnificus* populations by day 4 (Fig. 7). At 2 and 4°C, the numbers of *V. vulnificus* remained at this level through the 14 days of storage. At 0°C, there were additional 1- and 1.5-log decreases by days 8 and 10, respectively. Storage at 30°C for 3 days resulted in more than a 2-log increase. This increase in *V. vulnificus* numbers was reversed after oysters were

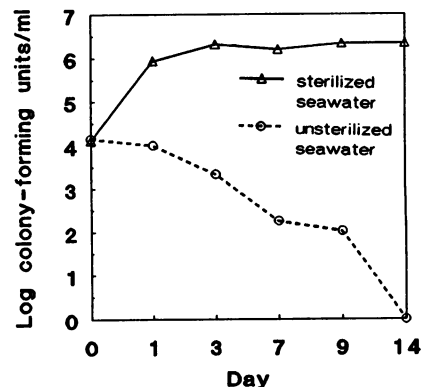


FIG. 6. Survival of a RIF-resistant strain of *V. vulnificus* (VV^{RIF}) in 20-ppt seawater incubated at 20°C. Mean values from duplicate flasks are shown.

transferred from 30 to 4°C and incubated for 24 h (Fig. 7), indicating that replicating *V. vulnificus*, like laboratory-grown cultures, is more susceptible to low storage temperatures than endogenous *V. vulnificus* present in oysters. These results emphasize the importance of proper storage of oyster shellstock following harvest and during shipment. These findings are in agreement with other studies (5, 7, 11) but conflict with the results of Murphy and Oliver (15), who reported a 3-log drop in *V. vulnificus* numbers when shellstock was stored at 22°C. In the latter study, *V. vulnificus* CVD713 was grown in the laboratory and fed to oysters rather than monitoring the endogenous *V. vulnificus* population. Perhaps the differences between studies are due to the inability of strain CVD713, which contains a *TnphoA* chromosomal insert, to compete with endogenous microbial flora or colonize oyster tissues. When oysters are allowed to take up laboratory-grown *V. vulnificus* strains from inoculated water, these strains, including CVD713, do not replicate in oysters like endogenous *V. vulnificus* and can be more easily removed by depuration (unpublished data). These findings show that endogenous populations of *V. vulnificus* differ from laboratory-grown cultures and are

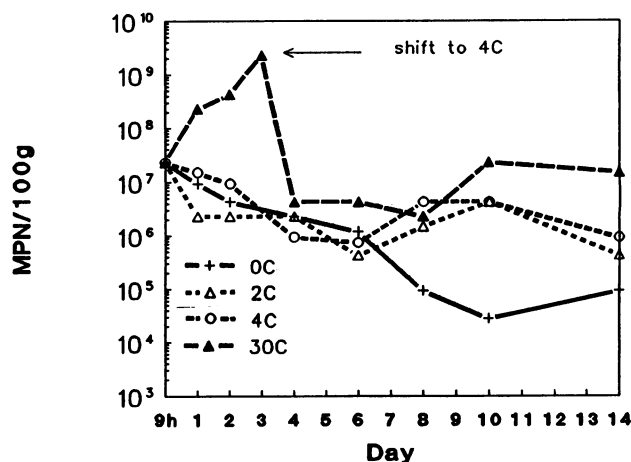


FIG. 7. Most probable numbers (MPN) of endogenous *V. vulnificus* organisms per 100 g of oyster meats during storage of shell stock at low (0 to 4°C) and abuse (30°C) temperatures. MPN were determined for 10 oysters at each sample point.

consistent with the results of studies on the adaptive responses of bacteria to environment factors (1, 8, 14, 17).

In summary, detailed studies over broad ranges of temperature and salinity demonstrate that temperatures outside the range of 13 to 22°C and salinities greater than 25 ppt reduce the survival of *V. vulnificus* in seawater. In addition, unidentified biological agents also contribute to the demise of *V. vulnificus* in unsterilized seawater. In oysters, elevated temperature promotes replication of *V. vulnificus* and survival is prolonged at low temperatures. These studies show that storage temperature of postharvest oyster shellstock is critical to controlling the levels of *V. vulnificus*. Also, investigators of future studies on *V. vulnificus* in oysters should be aware of the differences between endogenous populations and laboratory-grown cells.

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